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(54) Title: AN ENZYME WITH XYLANASE ACTIVITY

(57) Abstract

The present invention relates to an enzyme with xylanase activity, a cloned DNA sequence encoding the enzyme with xylanase activity, a method of producing the enzyme, an enzyme preparation comprising said enzyme with xylanase activity, a detergent composition comprising said xylanase, and the use of said enzyme and enzyme preparation for a number of industrial applications.

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TITLE: An enzyme with xylanase activity

FIELD OF INVENTION

The present invention relates to an enzyme with xylanase activity, a cloned DNA sequence encoding the enzyme with xylanase activity, a method of producing the enzyme, an enzyme composition comprising said enzyme with xylanase activity, and the use of said enzyme and enzyme preparation for a number of industrial applications.

BACKGROUND OF THE INVENTION

Xylan, a major component of plant hemicellulose, is a polymer of D-xylose linked by beta-1,4-xylosidic bonds. Xylan can be degraded to xylose and xylo-oligomers by acid or enzymatic hydrolysis. Enzymatic hydrolysis of xylan produces free sugars without the by-products formed with acid (e.g. furans).

Enzymes which are capable of degrading xylan and other plant cell wall polysaccharides are important for the feed and food industry. In the feed industri xylanases are primarily used as feed enhancers and for processing of feed. In the food industry xylanases are primarily used for baking, and in fruit and vegetable processing such as in wheat separation, fruit juice production or wine making, where their ability to catalyse the degradation of the backbone or side chains of the plant cell wall polysaccharide is utilised (Visser et al., in "Xylans and Xylanases", Elsevier Science publisher, 1991).

Other applications for xylanases are enzymatic breakdown of agricultural wastes for production of alcohol fuels, for hydrolysis of pentosans, manufacturing of dissolving pulps yielding cellulose, and bio-bleaching of wood pulp [*Detroym R.W.* In: Organic Chemicals from Biomass, (CRC Press, Boca Raton, FL, 1981) 19-41.; *Paice, M.G., and L. Jurasek.*, J. Wood Chem. Technol. 4: 187-198.; *Pommier, J.C., J.L. Fuentes, G. Goma.*, Tappi Journal (1989): 187-191.; *Senior, D.J., et al.*, Biotechnol. Letters 10 (1988):907-912].

WO 92/17573 discloses a substantially pure xylanase derived from the fungal species *H. insolens* and recombinant DNA encoding said xylanase. The xylanase is stated to be useful as a baking agent, a feed additive, and in the preparation of paper and pulp.

WO 92/01793 discloses a xylanase derived from the fungal species *Aspergillus tubigensis*. It is mentioned, but not shown that related xylanases may be derived from other filamentous fungi, examples of which are *Aspergillus*, *Disporotrichum*, *Penicillium*, *Neurospora*, *Fusarium* and *Trichoderma*. The xylanases are stated to be useful in the preparation of bread or animal feed, in breewing and in reducing viscosity or improving filterability of cereal starch.

Shei et al.(Biotech. and Bioeng. vol XXVII 553-538, 1985), and Fournier et al.(Biotech. and Bioeng. vol XXVII 539-546, 1985). describe purification and characterization of endoxylanases isolated from *A. niger*.

WO 91/19782 and EP 463 706 discloses xylanase derived from *Aspergillus niger* origin and the recombinant production thereof. The xylanase is stated to be useful for baking, brewing, in the paper making industry, and in the treatment of agricultural waste, etc.

Torronen, A et al. (Biotechnology 10:1461-1465, 1992) decribe cloning and characterization of two xylanases from Trichoderma reesei and Haas, H et al.(Gene 126:237-242, 1992) describe cloning of a xylanase from *Penicillum chrysogenum*.

WO 94/21785 discloses various xylanases isolated from *A. Aculeatus*.

SUMMARY OF THE INVENTION

The present inventors have surprisingly found that an enzyme exhibiting xylanase activity may be obtained from a strain of the genus *Myceliophtora*, more specifically *Myceliophtora thermophila*, and have succeeded in cloning a DNA sequence encoding said enzyme.

Accordingly, in a first aspect the invention relates to a

cloned DNA sequence encoding an enzyme exhibiting xylanase activity, which DNA sequence comprises:

(a) the xylanase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9978;
(b) the DNA sequence shown in positions 1-1128 in SEQ ID NO 1 or more preferably 79-1128 or its complementary strand;
(c) an analogue of the DNA sequence defined in (a) or (b) which is at least 70% homologous with said DNA sequence;
(d) a DNA sequence which hybridizes with the DNA sequence shown in positions 1-1128 in SEQ ID NO 1 at low stringency;
(e) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with the sequences of (b) or (d), but which codes for a polypeptide having the same amino acid sequence as the polypeptide encoded by any of these DNA sequences; or
(f) a DNA sequence which is a allelic form or fragment of the DNA sequences specified in (a), (b), (c), (d), or (e).

In a further aspect the invention relates to an isolated enzyme exhibiting xylanase activity selected from the group consisting of:

(a) a polypeptide encoded by the xylanase enzyme encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9978;
(b) a polypeptide comprising an amino acid sequence as shown in positions 27-375 of SEQ ID NO 2;
(c) an analogue of the polypeptide defined in (a) or (b) which is at least 70 % homologous with said polypeptide; and
(d) an allelic form or fragment of (a), (b) or (c).

In a still further aspect the invention provides a recombinant expression vector, which enables recombinant production of an enzyme of the invention. When using a heterologous host cell it is possible to make a highly purified

xylanase composition, characterized in being free from homologous impurities. This is highly advantageous for a number of industrial applications.

Finally the invention relates to an isolated substantially pure biological culture of the *Saccharomyces cerevisiae* strain DSM No. 9978 harboring a xylanase encoding DNA sequence (the xylanase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9978) obtained from a strain of the filamentous fungus *Myceliophthora thermophila* (it will be understood that any mutant of said *Saccharomyces* strain having retained the xylanase encoding capability is considered to be included in the present invention).

Comparison with prior art

A homology search with the xylanase gene of SEQ ID No 1 and the amino acid sequence of the SEQ ID No 2 xylanase of the invention against nucleotide and protein databases was performed. The homology search showed that the most related xylanase was a beta-1,4-xylanase from Cellulomonas fimi. The xylanase from Cellulomonas fimi belongs to family 10 of glycosyl hydrolases (Henrissat, B., Biochem. J. 280:309-316, 1991) which indicate that the xylanase of the invention also belongs to family 10 of glycosyl hydrolases. According to the method described in the "DETAILED DESCRIPTION OF THE INVENTION" the DNA homology of the xylanase of the invention against most prior art xylanases was determined using the computer program GAP. The xylanase gene with SEQ ID No 1 of the invention has only 55% DNA homology to the beta-1,4-xylanase from Cellulomonas (Gilkes N.R. et al., (1991) Eur J Biochem 202: 367-77) and only 56% DNA homology to xylanase I from Aspergillus aculeatus (WO 94/21785). This show that the xylanase of the invention indeed is distant from any known xylanases.

BRIEF DESCRIPTION OF DRAWINGS

Fig 1: the pH optimum for the xylanase,

Fig 2: the temperature optimum for the xylanase.

DEFINITIONS

Prior to discussing this invention in further detail, the following terms will first be defined.

"A cloned DNA sequence": The term "A cloned DNA sequence", refers to a DNA sequence cloned in accordance with standard cloning procedures used in genetic engineering to relocate a segment of DNA from its natural location to a different site where it will be reproduced. The cloning process involves excision and isolation of the desired DNA segment, insertion of the piece of DNA into the vector molecule and incorporation of the recombinant vector into a cell where multiple copies or clones of the DNA segment will be replicated.

The "cloned DNA sequence" of the invention may alternatively be termed "DNA construct" or "isolated DNA sequence".

"Obtained from": For the purpose of the present invention the term "obtained from" as used herein in connection with a specific microbial source, means that the enzyme is produced by the specific source, or by a cell in which a gene from the source have been inserted.

"An isolated polypeptide": As defined herein the term, "an isolated polypeptide" or "isolated xylanase", as used about the xylanase of the invention, is a xylanase or xylanase part which is essentially free of other non-xylanase polypeptides, e.g., at least about 20% pure, preferably at least about 40% pure, more preferably about 60% pure, even more preferably about 80% pure, most preferably about 90% pure, and even most preferably about 95% pure, as determined by SDS-PAGE.

The term "isolated polypeptide" may alternatively be termed "purified polypeptide".

"Homologous impurities": As used herein the term "homologous impurities" means any impurity (e.g. another polypeptide than the enzyme of the invention) which originate from the homologous cell where the enzyme of the invention is

originally obtained from. In the present invention the homologous cell may *e.g.* be a strain of *Myceliophtora thermophila*.

"xylanase encoding part": As used herein the term "xylanase encoding part" used in connection with a DNA sequence means the region of the DNA sequence which corresponds to the region which is translated into a polypeptide sequence. In the DNA sequence shown in SEQ ID NO 1 it is the region between the first "ATG" start codon ("AUG" codon in mRNA) and the following stop codon ("TAA", "TAG" or "TGA"). In others words this is the translated polypeptide.

The translated polypeptide comprises, in addition to the mature sequence exhibiting xylanase activity, an N-terminal signal sequence. The signal sequence generally guides the secretion of the polypeptide. For further information see Stryer, L., "Biochemistry" W.H., Freeman and Company/New York, ISBN 0-7167-1920-7.

In the present context the term "xylanase encoding part" is intended to cover the translated polypeptide and the mature part thereof.

DETAILED DESCRIPTION OF THE INVENTION

Cloned DNA sequence

In its first aspect the invention relates to a cloned DNA sequence encoding an enzyme exhibiting xylanase activity, which DNA sequence comprises

(a) the *xylanase* encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9978;

(b) the DNA sequence shown in positions 1-1128 in SEQ ID NO 1, or more preferably 79-1128, or its complementary strand;

(c) an analogue of the DNA sequence defined in (a) or (b) which is at least 70% homologous with said DNA sequence;

(d) a DNA sequence which hybridizes with the DNA sequence shown in positions 1-1128 in SEQ ID NO 1 at low stringency;

(e) a DNA sequence which, because of the degeneracy of the

genetic code, does not hybridize with the sequences of (b) or (d), but which codes for a polypeptide having the same amino acid sequence as the polypeptide encoded by any of these DNA sequences; or

(f) a DNA sequence which is an allelic form or fragment of the DNA sequences specified in (a), (b), (c), (d), or (e).

It is presently believed that the xylanase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in DSM 9978 is identical to the xylanase encoding part of the DNA sequence presented in SEQ ID NO 1.

Accordingly, the terms "the xylanase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in DSM 9978" and "the xylanase encoding part of the DNA sequence presented in SEQ ID NO 1" may be used interchangeably.

The DNA sequence may be of genomic, cDNA, or synthetic origin or any combination thereof.

The present invention also encompasses a cloned DNA sequence which encodes an enzyme exhibiting xylanase activity having the amino acid sequence set forth as the mature part of SEQ ID NO 2 (*i.e.* pos. 27-375), which DNA sequence differs from SEQ ID NO 1 by virtue of the degeneracy of the genetic code.

The DNA sequence shown in SEQ ID NO 1 and/or an analogue DNA sequence of the invention may be obtained from a microorganism such as a bacteria, a yeast or a filamentous fungus. Preferably it is obtained from a filamentous fungus and examples of suitable ones are given in the section "Microbial sources" (*vide infra*).

Alternatively, the analogous sequence may be constructed on the basis of the DNA sequence presented as the xylanase encoding part of SEQ ID No. 1, *e.g.* be a sub-sequence thereof and/or be constructed by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the xylanase encoded by the DNA sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid

sequence (*i.e.* a variant of the xylanase of the invention).

When carrying out nucleotide substitutions, amino acid changes are preferably of a minor nature, *i.e.* conservative amino acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification, such as a poly-histidine tract; an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids, such as arginine, lysine, histidine; acidic amino acids, such as glutamic acid and aspartic acid; polar amino acids, such as glutamine and asparagine; hydrophobic amino acids, such as leucine, isoleucine, valine; aromatic amino acids, such as phenylalanine, tryptophan, tyrosine; and small amino acids, such as glycine, alanine, serine, threonine, methionine. For a general description of nucleotide substitution, see *e.g.* Ford et al., (1991), Protein Expression and Purification 2, 95-107.

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide encoded by the cloned DNA sequence of the invention, and therefore preferably not subject to substitution may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (cf. *e.g.* Cunningham and Wells, (1989), Science 244, 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (*i.e.* xylanase) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photo affinity labelling (cf. *e.g.* de Vos et al., (1992), Science 255,

306-312; Smith et al., (1992), J. Mol. Biol. 224, 899-904; Wlodaver et al., (1992), FEBS Lett. 309, 59-64).

Polypeptides of the present invention also include fused polypeptides or cleavable fusion polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleic acid sequence (or a portion thereof) encoding another polypeptide to a nucleic acid sequence (or a portion thereof) of the present invention. Techniques for producing fusion polypeptides are known in the art, and include, ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator.

In a preferred embodiment the invention relates to a cloned DNA sequence, in which the DNA sequence encoding an enzyme exhibiting xylanase activity is obtainable from a microorganism, preferably a filamentous fungus, a yeast, or a bacteria.

In another preferred embodiment the invention relates to a cloned DNA sequence, in which the DNA sequence is isolated from or produced on the basis of a DNA library of the strain *of Myceliophthora*, in particular a strain of *M. thermophila*, especially *M. thermophila*, CBS 117.65.

In a further preferred embodiment the invention relates to a cloned DNA sequence, in which the DNA sequence is obtainable from a strain of *Aspergillus*, *Trichoderma*, *Fusarium*, *Humicola*, *Neocallimastix*, *Piromyces*, *Penicillium*, *Aureobasidium*, *Thermoascus*, *Paecilomyces*, *Talaromyces*, *Magnaporthe*, *Schizophyllum*, *Filibasidium*, *or a Cryptococcus*.

The DNA sequence of the invention, having the nucleotide sequence shown in SEQ ID NO 1, can be cloned from the strain *Saccharomyces cerevisiae* DSM No. 9978 using standard cloning techniques *e.g.* as described by Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY.

The DNA sequence of the invention can also be cloned by any general method involving

- cloning, in suitable vectors, a cDNA library from any organism expected to produce the xylanase of interest,
- transforming suitable yeast host cells with said vectors,
- culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the cDNA library,
- screening for positive clones by determining any xylanase activity of the enzyme produced by such clones, and
- isolating the enzyme encoding DNA from such clones.

A general isolation method of use in connection with the present invention has been disclosed in WO 93/11249 and WO 94/14953. A more detailed description of the screening method is given in a working example herein (*vide infra*).

Alternatively, the DNA encoding a xylanase of the invention may, in accordance with well-known procedures, conveniently be cloned from a suitable source, such as any of organisms mentioned in the section "Microbial Sources", by use of hybridization to synthetic oligonucleotide probes prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of or preferably be the xylanase encoding part of the nucleotide sequences presented as SEQ ID No. 1 or any suitable subsequence thereof, or the basis of the amino acid sequence SEQ ID NO 2.

Alternatively, the DNA sequence may be cloned by use of PCR primers prepared on the basis of the DNA sequence disclosed herein.

Homology of DNA sequences

The DNA sequence homology referred to above is determined as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation

penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the analogous DNA sequences referred to above exhibits a degree of identity preferably of at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 97% with the xylanase encoding part of the DNA sequence shown in SEQ ID No. 1.

Hybridization

The hybridization conditions referred to above to define an analogous DNA sequence as defined in d) above which hybridizes to the xylanase encoding part of the DNA sequence shown in SEQ ID NO 1, i.e. nucleotides 1-1128, under at least low stringency conditions, but preferably at medium or high stringency conditions are as described in detail below.

Suitable experimental conditions for determining hybridization at low, medium, or high stringency between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (Sodium chloride/Sodium citrate, Sambrook et al. 1989) for 10 min, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a concentration of 10ng/ml of a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS at least 55°C (low stringency), more preferably at least 60°C (medium stringency), still more preferably at least 65°C (medium/high stringency), even more preferably at least 70°C (high stringency), and even more preferably at least 75°C (very high stringency).

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a x-ray film.

It has been found that it is possible to theoretically predict whether or not two given DNA sequences will hybridize

under certain specified conditions.

Accordingly, as an alternative to the above described experimental method the determination whether or not an analogous DNA sequence will hybridize to the nucleotide probe described above, can be based on a theoretical calculation of the Tm (melting temperature) at which two heterologous DNA sequences with known sequences will hybridize under specified conditions (e.g. with respect to cation concentration and temperature).

In order to determine the melting temperature for heterologous DNA sequences (Tm(hetero)) it is necessary first to determine the melting temperature (Tm(homo)) for homologous DNA sequences.

The melting temperature Tm(homo) between two fully complementary DNA strands (homoduplex formation) may be determined by use of the following formula, Tm(homo) = 81.5°C + 16.6(log M) + 0.41(%GC) - 0.61 (% form) - 500/L ("Current protocols in Molecular Biology". John Wiley and Sons, 1995), wherein

"M" denotes the molar cation concentration in wash buffer,

"%GC" % Guanine (G) and Cytosine (C) of total number of bases in the DNA sequence,

"% form" % formamid in the wash buffer, and

"L" the length of the DNA sequence.

Using this formula and the experimental wash conditions given above, Tm(homo) for the homoduplex formation of the nucleotide probe corresponding to the DNA sequence shown in SEQ ID NO 1, i.e. nucleotides 1-1128 is:

Tm(homo) = 81.5 + 16.6 (log 0.30) + 0.41(66) - 0.61(0) - (500/1128)

Tm(homo) = 99 °C

"M": 2 X SSC corresponds to a cation conc. of 0.3M.

"%GC" The %GC in SEQ ID No 1 is 66%.

"% form": There is no formamid in the wash buffer.

"L": The length of SEQ ID No 1 is 1128.

The Tm determined by the above formula is the Tm of a homoduplex formation (Tm(homo)) between two fully complementary DNA sequences. In order to adapt the Tm value to that of two

heterologous DNA sequences, it is assumed that a 1% difference in nucleotide sequence between the two heterologous sequences equals a 1°C decrease in Tm ("Current protocols in Molecular Biology". John Wiley and Sons, 1995). Therefore, the Tm(hetero) for the heteroduplex formation is found by subtracting the homology % difference between the analogous sequence in question and the nucleotide probe described above from the Tm(homo). The DNA homology percentage to be subtracted is calculated as described herein (*vide supra*).

With the experimental conditions above and a wash temperature of 55°C (low stringency) , an analogous sequence with 56% *(100 - (99(Tm(homo) - 55) = 56%)* homology will be considered to hybridize to the nucleotide probe described above. With the more preferably wash temperature at 65°C (medium stringency) an analogous sequence with 66% *(100 - (99(Tm(homo) - 65) = 66%)* homology will hybridize *etc.*

In a further aspect the invention relates to an enzyme exhibiting xylanase activity defined by properties (a)-(d) referred to above.

Homology to amino acid sequences

The polypeptide homology referred to above (property (c)) of the polypeptide of the invention is determined as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the mature part of a polypeptide encoded by an analogous DNA sequence of the invention exhibits a degree of identity preferably of at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, and especially at least 97% with the mature part of the amino acid sequence shown in SEQ ID NO 2, *i.e.* position 27-

375 in SEQ ID NO 2.

In a preferred embodiment the invention relates to an enzyme exhibiting xylanase activity defined by properties (a)-(d) referred to above which further has a pH optimum in the range of 4-6, measured at 30°C, more preferably a pH optimum in the range of 4.5-5.5, measured at 30°C; and/or a molecular mass of 43 ± 10 kDa, as determined by SDS-PAGE, more preferably a molecular mass of 43 ± 5 kDa, as determined by SDS-PAGE, and even more a molecular mass of 43 ± 3 kDa, as determined by SDS-PAGE; and/or a temperature optimum in the range between 65°C to 75°C, measured at pH 5, more preferably a temperature optimum in the range between 67°C to 73°C, measured at pH 5, even more preferably a temperature optimum in the range between 68.5°C to 71.5°C, measured at pH 5; and/or a specific activity in the range between 425-575 µmol/min/ mg enzyme, measured with birch xylan as substrate at 30°C, more perferably a specific activity in the range between 450-525 µmol/min/ mg enzyme, measured with birch xylan as substrate at 30°C, and even more preferably a specific activity in the range between 465-510 µmol/min/ mg enzyme, measured with birch xylan as substrate at 30°C.

The pH optimum was measured with birch xylan (Roth) as substrate in a 0.1M citrate/phosphate buffer at 30°C. For further details reference is made to a working example herein (*vide infra*).

The molecular mass is measured by SDS-PAGE electrophoresis as further described in the "Material and Methods" section (*vide infra*).

The temperature optimum was measured with birch xylan (Roth) as substrate in a 0.1M citrate/phosphate buffer at pH 5. For further details reference is made to a working example herein (*vide infra*).

The specific activity was measured by carrying out incubations at substrate concentrations (S) ranging from 0.05 to 1.5% (birch xylan), measure the reaction rate (v), picture S/v as a function of S, carry out linear regression analysis, finding the

slope (=1/Vmax) and the intercept (Km/Vmax) and calculating Km and the specific activity (=Vmax/E), where E is the amount of enzyme added.

For further details reference is made to a working example herein (*vide infra*).

The present invention is also directed to xylanase variants which have an amino acid sequence which differs by no more than three amino acids, preferably by no more than two amino acids, and more preferably by no more than one amino acid from the mature part of the amino acid sequence set forth in SEQ ID NO 2.

Microbial Sources

In a preferred embodiment, the DNA sequence encoding the xylanase is derived from a strain of *Myceliophthora*, especially a strain of *Myceliophthora thermophila*.

It is at present contemplated that a DNA sequence encoding an enzyme homologous to the enzyme of the invention, *i.e.* an analogous DNA sequence, may be obtained from other microorganisms. For instance, the DNA sequence may be derived by similarly screening a cDNA library of another microorganism, in particular a fungus, such as a strain of an *Aspergillus sp.*, in particular a strain of *A. aculeatus* or *A. niger*, a strain of *Trichoderma sp.*, in particular a strain of *T. reesei*, *T. viride*, *T. longibrachiatum*, *T. harzianum* or *T. koningii* or a strain of a *Fusarium sp.*, in particular a strain of *F. oxysporum*, or a strain of a *Humicola sp.*, or a strain of a *Neocallimastix sp.*, a *Piromyces sp.*, a *Penicillium sp.*, an *Aureobasidium sp.*, a *Thermoascus sp.*, a *Paecilomyces sp.*, a *Talaromyces sp.*, a *Magnaporthe sp.*, a *Schizophyllum sp.*, a *Filibasidium sp.*, or a *Cryptococcus sp.*

The expression plasmid pYES 2.0 comprising the full length DNA sequence encoding the xylanase of the invention has been transformed into a strain of the *S. cerevisiae* which was deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Deutshe Sammlung von Mikro-

organismen und Zellkulturen GmbH.

Deposit date : 11.05.95
Depositor's ref. : NN049011
DSM designation : *Saccharomyces cerevisiae* DSM No. 9978

Expression vectors

In another aspect, the invention provides a recombinant expression vector comprising the cloned DNA sequence of the invention.

The expression vector of the invention may be any expression vector that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the expression vector, the DNA sequence encoding the xylanase should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the xylanase, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., (1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY).

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (*McKnight et al.*, The EMBO J. 4 (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral a-amylase,

Aspergillus niger acid stable a-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase or *Aspergillus nidulans* acetamidase.

Host cells

In yet another aspect the invention provides a host cell comprising the cloned DNA sequence of the invention and/or the recombinant expression vector of the invention.

Preferably, the host cell of the invention is a eukaryotic cell, in particular a fungal cell such as a yeast or filamentous fungal cell. In particular, the cell may belong to a species of *Trichoderma*, preferably *Trichoderma harzianum* or *Trichoderma reesei*, or a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger*, or a species of *Fusarium*, most preferably *Fusarium graminearum* or *Fusarium cerealis*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (Novo Nordisk A/S). The host cell may also be a yeast cell, *e.g.* a strain of *Saccharomyces*, in particular *Saccharomyces* cerevisae, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp., *Pichia* sp., *Yarrowia* sp., such as *Yarrowia lipolytica*, or *Kluyveromyces* sp., such as *Kluyveromyces lactis*.

Method of producing xylanase

The present invention provides a method of producing an isolated enzyme according to the invention, wherein a suitable host cell, which has been transformed with a DNA sequence encoding the enzyme, is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

When an expression vector comprising a DNA sequence encoding the enzyme is transformed into a heterologous host cell

it is possible to enable heterologous recombinant production of the enzyme of the invention.

Thereby it is possible to make a highly purified xylanase composition, characterized in being free from homologous impurities.

In the present invention the homologous host cell may be a strain of *Myceliophtora thermophila*.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed xylanase may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Enzyme compositions

In a still further aspect, the present invention relates to an enzyme preparation useful for the degradation of plant cell wall components, said preparation being enriched in an enzyme exhibiting xylanase activity as described above. In this manner a boosting of the cell wall degrading ability of the enzyme preparation can be obtained.

The enzyme composition having been enriched with an enzyme of the invention may e.g. be an enzyme preparation comprising multiple enzymatic activities, in particular an enzyme preparation comprising multiple plant cell wall degrading enzymes such as Biofeed+®, Energex®, Viscozym®, Pectinex®, Pectinex Ultra SP®, (all available from Novo Nordisk A/S). In the present context, the term "enriched" is intended to indicate that the xylanase activity of the enzyme preparation has been increased, e.g. with an enrichment factor of 1.1, conveniently due to addition of an enzyme of the invention prepared by the method described above.

Alternatively, the enzyme preparation enriched in an enzyme exhibiting xylanase activity may be one which comprises an

enzyme of the invention as the major enzymatic component, e.g. a mono-component enzyme preparation.

The enzyme preparation may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry preparation. For instance, the enzyme preparation may be in the form of a granulate or a microgranulate. The enzyme to be included in the preparation may be stabilized in accordance with methods known in the art.

The enzyme preparation of the invention may, in addition to a xylanase of the invention, contain one or more other enzymes, for instance those with xylanolytic, or pectinolytic activities such as α-arabinosidase, α-glucoronisidase, β-xylosidase, xylan acetyl esterase, arabinanase, rhamnogalacturonase, pectin acetylesterase, galactanase, pectin lyase, pectate lyase, glucanase, pectin methylesterase. The additional enzyme(s) may be producible by means of a microorganism belonging to the genus *Aspergillus*, preferably *Aspergillus niger*, *Aspergillus aculeatus*, *Aspergillus awamori* or *Aspergillus oryzae*, or *Trichoderma*, or *Humicola insolens*. Examples are given below of preferred uses of the enzyme preparation of the invention. The dosage of the enzyme preparation of the invention and other conditions under which the preparation is used may be determined on the basis of methods known in the art. In general terms, the enzyme is to be used in an efficient amount for providing the desired effect.

The enzyme preparation according to the invention may be useful for at least one of the following purposes.

Degradation or modification of plant material

The enzyme preparation according to the invention is preferably used as an agent for degradation or modification of plant cell walls or any xylan-containing material originating from plant cells walls due to the high plant cell wall degrading activity of the xylanase of the invention.

The xylanase of the invention hydrolyse β-1,4 linkages in xylans. Xylans are polysaccharides having a backbone composed of β-1,4 linked xylose. The backbone may have different sidebranches, like arabinose, acetyl, glucuronic acid and 4-methylglucuronic

acid sidebranches. The composition and number of sidebranches vary according to the source of the xylan. Arabinose sidebranches dominate in xylans from cereal endosperm, whereas xylans from hard wood contain relatively more acetyl and glucuronic acid substituents (Michael P. Coughlan and Geoffrey P. Hazlewood. Biotechnol.Appl. Biochem. 17 : 259-289 (1993). Xylan originating from red algae contains a mixture of β-1,4 and β-1,3 linked xylose in the backbone, this type of xylan is degradable by xylanases to varying extent due to the 1,4-links in the backbone.

The degradation of xylan by xylanases is facilitated by full or partial removal of the sidebranches. Acetyl groups can be removed by alkali, or by xylan acetyl-esterases, arabinose sidegroups can be removed by a mild acid treatment or by alpha-arabinosidases and the glucuronic acid sidebranches can be removed by alpha-glucuronisidases. The oligomers with are released by the xylanase or by a combination of xylanases and sidebranch-hydrolysing enzymes as mentioned above can be further degraded to free xylose by beta-xylosidases.

The xylanase of the present invention can be used without other xylanolytic enzymes or with limited activity of other xylanolytic enzymes to degrade xylans for production of oligosaccharides. The oligosaccharides may be used as bulking agents, like arabinoxylan oligosaccharides released from cereal cell wall material, or of more or less purified arabinoxylans from cereals.

The xylanase of the present invention can be used in combination with other xylanolytic enzymes to degrade xylans to xylose and other monosaccharides. The released xylose may be converted to other compounds like furanone flavours.

The xylanase of the present invention may be used alone or together with other enzymes like a glucanase to improve the extraction of oil from oil-rich plant material, like corn-oil from corn-embryos.

The xylanase of the present invention may be used for separation of components of plant cell materials, in particular of cereal components such as wheat components. Of particular interest is the separation of wheat into gluten and starch, i.e. components of considerable commercial interest. The separation process may

be performed by use of methods known in the art, conveniently a so-called batter process (or wet milling process) performed as a hydroclone or a decanter process. In the batter process, the starting material is a dilute pumpable dispersion of the plant material such as wheat to be subjected to separation. In a wheat separation process the dispersion is made normally from wheat flour and water. Wheat separation is e.g. disclosed in WO95/23514.

The xylanase of the invention may also be used in the preparation of fruit or vegetable juice in order to increase yield, and in the enzymatic hydrolysis of various plant cell wall-derived materials or waste materials, e.g. from paper production, or agricultural residues such as wheat-straw, corn cobs, whole corn plants, nut shells, grass, vegetable hulls, bean hulls, spent grains, sugar beet pulp, olive pulp, and the like.

The plant material may be degraded in order to improve different kinds of processing, facilitate purification or extraction of other component than the xylans like purification of beta-glucan or beta-glucan oligomers from cereals, improve the feed value, decrease the water binding capacity, improve the degradability in waste water plants, improve the conversion of e.g. grass and corn to ensilage, etc.

Also, the xylanase of the invention may be used in modifying the viscosity of plant cell wall derived material. For instance, the xylanase may be used to reduce the viscosity of feed containing xylan, to promote processing of viscous xylan containing material as in wheat separation, and to reduce viscosity in the brewing process.

Preparation of dough or baked product

The xylanase of the present invention may be used in baking so as to improve the development, elasticity and/or stability of dough and/or the volume, crumb structure and/or anti-staling properties of the baked product. The xylanase may be used for the preparation of dough or baked products prepared from any type of flour or meal (e.g. based on rye, barley, oat, or maize), particularly in the preparation of dough or baked products made from wheat or comprising substantial amounts of wheat. The baked

products produced with an xylanase of the invention includes bread, rolls, baquettes and the like. For baking purposes the xylanase of the invention may be used as the only or major enzymatic activity, or may be used in combination with other enzymes such as a lipase, an amylase, an oxidase (e.g. glucose oxidase, peroxidase), a laccase and/or a protease.

Animal Feed Additives

The xylanase of the present invention may be used for modification of animal feed and may exert their effect either *in vitro* (by modifying components of the feed) or *in vivo*. The xylanase is particularly suited for addition to animal feed compositions containing high amounts of arabinoxylans and glucuronoxylans, e.g. feed containing cereals such as barley, wheat, rye or oats or maize. When added to feed the xylanase significantly improves the *in vivo* break-down of plant cell wall material partly due to a reduction of the intestinal viscosity (Bedford et al., 1993), whereby a better utilization of the plant nutrients by the animal is achieved. Thereby, the growth rate and/or feed conversion ratio (i.e. the weight of ingested feed relative to weight gain) of the animal is improved. The xylanases may be used in combination with other enzymes such as phytase or galactanase.

Paper and pulp industry

The xylanase of the present invention may be used in the paper and pulp industry, inter alia in the bleaching process to enhance the brightness of bleached pulps whereby the amount of chlorine used in the bleaching stages may be reduced, and to increase the freeness of pulps in the recycled paper process (Eriksson, K.E.L., Wood Science and Technology 24 (1990): 79-101; Paice, et al., Biotechnol. and Bioeng. 32 (1988): 235-239 and Pommier et al., Tappi Journal (1989): 187-191). Furthermore, the xylanase may be used for treatment of lignocellulosic pulp so as to improve the bleachability thereof. Thereby the amount of chlorine needed to obtain a satisfactory bleaching of the pulp may be reduced. The treament of lignocellulosic pulp may, e.g., be

performed as described in WO 93/08275, WO 91/02839 and WO 92/03608.

Beer brewing

The xylanase of the present invention may be used in beer brewing, in particular to improve the filterability of wort e.g. containing barley and/or sorghum malt. The xylanase may be used in the same manner as pentosanases conventionallly used for brewing, e.g. as described by Viëtor et al., 1993 and EP 227 159. Furthermore, the xylanase may be used for treatment of brewers spent grain, i.e. residuals from beer wort production containing barley or malted barley or other cereals, so as to improve the utilization of the residuals for, e.g., animal feed.

The invention is described in further detail in the following examples which are not in any way intended to limit the scope of the invention as claimed.

MATERIALS AND METHODS

Deposited organisms

Saccharomyces cerevisiae DSM 9978 containing the plasmid comprising the full length DNA sequence, coding for the xylanase of the invention, in the shuttle vector pYES 2.0.

Other strains

Myceliophthora thermophila CBS No. 117.65 comprises the xylanase encoding DNA sequence of the invention.

Yeast strain: The *Saccharomyces cerevisiae* strain used was W3124 (MATa; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-1137; prc1::HIS3; prb1:: LEU2; cir+).

E. coli strain: DH10B (Life Technologies)

Plasmids

The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The construction of pHD414 is further described in WO 93/11249.

pYES 2.0 (Invitrogen)

pA2X144 (See example 1)

General molecular biology methods

Unless otherwise mentioned the DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990).

Enzymes for DNA manipulations were used according to the specifications of the suppliers.

Enzymes for DNA manipulations

Unless otherwise mentioned all enzymes for DNA manipulations, such as e.g. restiction endonucleases, ligases etc., are obtained from New England Biolabs, Inc.

Expression cloning in yeast

Expression cloning in yeast was done as comprehensively described by H. Dalboege et al. (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953), which are hereby incorporated as reference.

All individual steps of Extraction of total RNA, cDNA synthesis, Mung bean nuclease treatment, Blunt-ending with T4 DNA polymerase, and Construction of libraries was done according to the references mentioned above.

Fermentation procedure of *Myceliophtora thermophila* CBS No. 117.65 for mRNA isolation

Myceliophtora thermophila CBS No. 117.65 was inoculated from a plate with outgrown mycelium into a shake flask containing 100 ml cellulose-containing medium PD liquid broth (24g potato dextrose broth, Difco 0549, deionized water up to 1000ml; autoclave (121°C for 15-20 min))

The culture was fermented at 26°C for 5 days. The

resulting culture broth was filtered through miracloth and the mycelium was frozen down in liquid nitrogen.

mRNA was isolated from mycelium from this culture as described in (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953).

Identification of positive yeast clones

Identification of positive yeast clones (i.e. clones which comprise a gene encoding for xylanase activity) was done as described below.

The yeast tranformants was plated on SC agar containing 0.1% AZCL xylan (Megazyme, Australia) and 2% Galactose and incubated for 3-5 days at 30 C.

Xylanase positive colonies are identified as colonies surrounded by a blue halo.

Isolation of a cDNA gene for expression in *Aspergillus*

A xylanase-producing yeast colony was inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

DNA was isolated according to WO 94/14953 and dissolved in 50 ml water. The DNA was transformed into *E. coli* by standard procedures. Plasmid DNA was isolated from *E. coli* using standard procedures, and analyzed by restriction enzyme analysis. The cDNA insert was excised using appropriate restriction enzymes and ligated into an Aspergillus expression vector.

Transformation of *Aspergillus oryzae* or *Aspergillus niger*

Protoplasts may be prepared as described in WO 95/02043, p. 16, line 21 - page 17, line 12, which is hereby incorporated by reference.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM $CaCl_2$). Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH

29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation is stored as a defined transformant.

Test of *A. oryzae* transformants

Each of the transformants were inoculated in 10 ml of YPM (cf. below) and propagated. After 2-5 days of incubation at 30°C, the supernatant was removed. The xylanolytic activity was identified by applying 10 µl supernatant to 4 mm diameter holes punched out in agar plates containing 0.2% AZCLÔ birch xylan (Megazyme®, Australia). Xylanolytic activity is then identified as a blue halo.

Fed batch fermentation

Fed batch fermentation was performed in a medium comprising maltodextrin as a carbon source, urea as a nitrogen source and yeast extract. The fed batch fermentation was performed by inoculating a shake flask culture of *A. oryzae* host cells in question into a medium comprising 3.5% of the carbon source and 0.5% of the nitrogen source. After 24 hours of cultivation at pH 7.0 and 34°C the continuous supply of additional carbon and nitrogen sources were initiated. The carbon source was kept as the limiting factor and it was secured that oxygen was present in excess. The fed batch cultivation was continued for 4 days.

Isolation of the DNA sequence shown in SEQ ID No. 1

The xylanase encoding part of the DNA sequence shown in SEQ ID No. 1 coding for the xylanase of the invention can be

obtained from the deposited organism *Saccharomyces cerevisiae* DSM 9978 by extraction of plasmid DNA by methods known in the art (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY).

Characterization of an enzyme of the invention

The molecular weight of the xylanase enzyme was determined using 4 to 20 % SDS-PAGE precasted plates from Novex Tm. Molecular weight of the protein was determined under reducing conditions according to standard procedure.

Isoelectric focusing, and Commassie and silver staining. Reference is here made to WO 94/21785.

The activities of the enzymes are measured either by the release of reducing sugars from birch xylan (available from Roth, Karlsruhe, Germany) or by the release of blue colour from AZCL-birch xylan from MegaZyme.

0.5ml 0.4% AZCL-substrate suspension is mixed with 0.5ml 0.1M citrate/phosphate buffer of optimal pH and 10 µl of a suitably diluted enzyme solution is added. Incubations are carried out in Eppendorf Thermomixers for 15 minutes at 30°C (if not otherwise specified) before placing in an ice- bath and cold centrifugation. Enzyme incubations are carried out in triplicate. A blank is produced in which enzyme is added but immediately placed on icebath. After centrifugation the absorbance of the supernatant is measured in microtiter plates at 620 nm and the blank is subtracted.

0.5% solutions of birch xylan (Roth) are made in 0.1M citrate/phosphate of the optimal pH, (if not otherwise specified) 10µl enzyme suitably diluted solutions are added to 1ml of substrate, incubations are carried out at 30°C for 15 minutes before heat-inactivation at 100°C for 20 minutes. Reducing sugars are determined by reaction, in microtiter plates, with a PHBAH reagent comprising 0.15 g of para hydroxy benzoic acid hydrazide (Sigma H-9882), 0.50 g of potassium-sodium tartrate (Merck 8087) and 2% NaOH solution up to 10.0 ml. Results of blanks are subtracted. Xylose is used as a standard.

pH and temperature optimums are measured on the above

mentioned substrates. 0.1M citrate/phosphate buffers of varying pH are used for determination of pH optimum. 0.1M citrate/phosphate buffers at optimal pH is used for reaction at different temperatures for 15 min. in order to determine the temperature optimum.

Km and specific activity are measured by carrying out incubations at substrate concentrations (S) ranging from 0.05 to 1.5% (birch xylan), measure the reaction rate (v), picture S/v as a function of S, carry out linear regression analysis, finding the slope (=1/Vmax) and the intercept (Km/Vmax) and calculating Km and the specific activity (=Vmax/E), where E is the amount of enzyme added.

Determination of FXU (W) (endo-xylanase activity)

The endo-xylanase activity is determined by an assay, in which the xylanase sample is incubated with a remazol-xylan substrate (4-O-methyl-D-glucurono-D-xylan dyed with Remazol Brilliant Blue R, Fluka), pH 6.0 at a substrate concentration of 0.45% (w/v) and an enzyme concentration of 0.011-0.057. The incubation is performed at 50°C for 30 min. The background of non-degraded dyed substrate is precipitated by ethanol. The remaining blue colour in the supernatant is determined spectrophotometrically at 585 nm and is proportional to the endoxylanase activity. The endoxylanase activity of the sample is determined relatively to an enzyme standard (Biofeed Wheat alias SP628) available upon request from Novo Nordisk A/S, Denmark.

Media

YPD: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% glucose (sterile filtered) added.

YPM: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% maltodextrin (sterile filtered) added.

10 x Basal salt: 75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA: 100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan, H_2O ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

SC-agar: SC-URA, 20 g/l agar added.

SC-variant agar: 20 g agar, 20 ml 10 x Basal salt, H_2O ad 900 ml, autoclaved

AZCL xylan (Megazyme, Australia)

PEG 4000 (polyethylene glycol, molecular weight = 4,000) (BDH, England)

EXAMPLES

EXAMPLE 1

Cloning and expression of a xylanase from *Myceliophthora thermophila* CBS No. 117.65

mRNA was isolated from *Myceliophthora thermophila*, CBS No. 117.65, grown in cellulose-containing fermentation medium (*vide supra*) with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. A library from *M. thermophila*, CBS No. 117.65, consisting of approx. $9x10^5$ individual clones was constructed in *E. coli* as described with a vector background of 1%. Plasmid DNA from some of the pools was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were obtained from each pool.

Xylanase-positive colonies were identified and isolated on SC-agar plates with the AZCL xylan assay. cDNA inserts were amplified directly from the yeast colonies and characterized as described in the Materials and Methods section above.

The DNA sequence of the cDNA encoding the xylanase is

shown in SEQ ID No. 1 and the corresponding amino acid sequence is shown in SEQ ID No. 2. In SEQ ID No. 1 DNA nucleotides from No 1 to No. 1128 define the xylanase encoding region.

The part of the DNA sequence in SEQ ID NO 1, which is encoding the mature part of the xylanase is position 79 to 1128, which correspond to amino acid position 27-375 in SEQ ID NO 2.

The cDNA is obtainable from the plasmid in DSM 9978.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the xylanase in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the xylanase gene was purified. The gene was subsequently ligated to pHD414, digested with appropriate restriction enzymes, resulting in the plasmid pA2X144.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described above.

Test of *A. oryzae* transformants

Each of the transformants were tested for enzyme activity as described above. Some of the transformants had xylanase activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of the xylanase in *Aspergillus oryzae*.

EXAMPLE 2

Purification of recombinant xylanase from *M. thermophlium*

The culture supernatant from the fermentation of *Aspergillus oryzae* expressing the recombinant enzyme is centrifuged and filtered through a 0.2μm filter to remove the mycelia. 250 ml of the filtered supernatant is ultrafiltered in a Filtron ultracette or Amicon ultrafiltration device with a 10kDa membrane and at the same time the buffer is changed to 25 mM Tris-HCl pH 8.0 in two successive rounds of ultrafiltration in the same device. The resulting 40ml sample is loaded at 1.5 ml/min onto a Pharmacia HR16/20 Fast Flow Q Sepharose anion exchange column equilibrated in 25mM Tris-HCl pH 8.0. After the sample has been

applied, the column is washed with two column volumes 25mM Tris-HCl pH 8.0 and bound proteins are eluted with a linear increasing NaCl gradient from 0 to 0.5M NaCl in 25 mM Tris-HCl pH 8.0. Fractions are tested for xylanase activity on AZCL-xylan and fractions containing the activity are pooled. The xylanase elutes at approximately 0.25M NaCl.

The buffer in the pooled fractions is changed to 10mM Phosphate buffer pH 6.8 and loaded at 1ml/min onto a Pharmacia XK26 BIO-GEL® HTP hydroxylapatite column equilibrated in 10mM Phosphate buffer pH 6.8. The column is washed in two column volumes of the same buffer and bound proteins are eluted with a linear increasing gradient from 10mM phosphate buffer to 0.5M phosphate buffer pH 6.8. Fractions are tested for xylanase activity and the xylanase elutes at approximately 100mM phosphate buffer. The fractions containing the xylanase activity are pooled. The buffer in the pooled fractions is changed into 25mM Tris-HCl pH 8.0 and the sample is loaded at 1ml/min onto a Pharmacia Mono Q HR10/10 column. After washing in two column volumes of 25mM Tris-HCl pH 8.0 the bound proteins are eluted with a linear increasing NaCl gradient from 0 to 0.4M NaCl in 25mM Tris-HCl pH 8.0. The xylanase activity elutes at approximately 0.2M NaCl and fractions containing the activity are pooled.

Protein concentration is determined by use of the "Bio-Rad protein assay" in accordance with the Manufactures (Bio-Rad Laboratories GmbH) recommendations.

EXAMPLE 3

Characterization of xylanase

The xylanase was characterized as described in Materials and Methods and the main results are apparent from the table 1 below:

	Xyl
Mw	43 kDa
pI	4.55 &5.5
Km	0.2-0.3%

Specific activity	470-505 µmol/min/mg enzyme

Table 1:

Mw was determined by SDS-PAGE.

pH and temperature optimum

The pH and temperature optimum of the enzyme can be seen in Fig. 1 and 2, respectively.

Km and specific activity

The Km and specific activity for xylanase were determined as described in the Materials and Methods section above at the optimal pH. The standard deviations on 1/Vmax and Km/Vmax obtained from the linear regression analysis were used to calculate the intervals for the enzymes apparent from the above table.

Aminoterminal sequence

Aminoterminal analysis was determined by using Edman degradation with Applied Biosystem equipment (ABI 473A protein sequencer, Applied Biosytem, USA) carried out as described by manufacturer.

N-terminal sequence(s):

For the xylanase of the invention having the amino acid sequence shown in SEQ ID NO 2 the N-terminal sequence is:

N-terminal Gly-Pro-His-Thr-Gly-Leu-

The N-terminal amino acid Gly is position 27 in SEQ ID NO 2. This indicates the mature xylanase enzyme of the invention starts at position 27 in SEQ ID No 2. Consequently the mature sequence is from 27-346 in SEQ ID no 2.

EXAMPLE 4

Use of xylanase from *Myceliophthora thermophila* in Baking

Materials and methods

FXU(W) - xylanase activity
Was determined as described above.

Preparation of bread in mini scale
White bread were prepared from the following basic recipe:

BASIC RECIPE	
Flour (Meneba)	100 % (10g)
Water	57 %
Yeast	4 %
Salt	1.5 %
Sugar	1.5 %
Ascorbic acid	30 ppm

The wheat flour was of the type termed "Manitoba" supplied by "Valsemøllerne", Denmark, October 1995.

BAKING PROCEDURE

Mixing (10 g mixograph)	2.5 min
Dough evaluation	4 min
Fermentation at 28°C	25 min
Sheeting/moulding/panning	2 min
Fermentation of breads (32°C, 85% RH)	45 min
Baking (230°C)	17 min

Evaluation of dough and baked products

Properties of the dough and baked products were determined as follows:

Loaf specific volume: the volume of a loaf is measured by means of glass bead displacement (analogous to the traditional rape seed displacement method). The specific volume is calculated as volume ml per g bread. The specific volume of the control (without enzyme) is defined as 100. The relative specific volume index is calculated as:

$$\text{Specific vol. index} = \frac{\text{specific volume of loaf}}{\text{specific volume of control loaf}} * 100$$

The figures shown in the table are mean values of at least 3 replicates.

The dough stickiness is evaluated manually according to the following scale:

Dough stickiness	almost liquid	1
	too sticky	2
	slightly sticky	3
	nice soft	3.5
	normal	4
	dry	5

Xylanase

The enzyme used was a xylanase of the invention from *Myceliophthora thermophila*. The enzyme was recombinantly expressed in A. oryzae an purified as described in Example 2 above. The enzyme was dispersed in water before being added to the baking ingredient mix. All tests were carried out in at least triplicate. The average results obtained are shown in Table 2.

Statistical analysis

All results (specific volume) were analyzed by means of ANOVA in Statgraphics release 7.0, with treatments variable and with days as block criteria. Multiple ranges test (LSD means) was used for grouping of treatments into significantly different groups (α=0.05).

Table 2

FXU(W)/kg Flour	0	50	80	120	200	400
SP volume	4.1	4.2	4.1	4.2	4.35	4.3

Table 2

Effect of Xylanase from *Myceliophthora thermophila* on specific loaf volume as a function of dosage in FXU(W) / kg flour.

At dosages ≥ 200 FXU(W)/kg, the specific volume was significantly different from the reference volume on a 5% level of significance. There seems to be an optimum around 200 FXU(W) and thus the xylanase of the invention when used for baking is preferably used in a dosage of 10-500 FXU (W)/kg of flour.

At all dosages the dough stickiness was acceptable, i.e the score in the dough stickiness scale shown above was above 3 in all cases.

SEQUENCE LISTING

SEQ ID No. 1 shows the DNA sequence of the full-length DNA sequence comprised in the DNA construct transformed into the deposited *Saccharomyces cerevisiae* DSM 9978.

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1128 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Myceliophthora thermophila
(B) STRAIN: CBS 117.65

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1125

```
ATG CAT CTC TCC TCG TCT CTC CTC CTC CTC GCC GCC TTG CCC CTG GGC        48
Met His Leu Ser Ser Ser Leu Leu Leu Leu Ala Ala Leu Pro Leu Gly
  1               5                  10                  15

ATC GCC GGC AAG GGC AAG GGC CAC GGC CAC GGC CCC CAT ACC GGG CTC        96
Ile Ala Gly Lys Gly Lys Gly His Gly His Gly Pro His Thr Gly Leu
            20                  25                  30

CAC ACC CTC GCC AAG CAG GCC GGC CTC AAG TAC TTC GGC TCT GCC ACC       144
His Thr Leu Ala Lys Gln Ala Gly Leu Lys Tyr Phe Gly Ser Ala Thr
        35                  40                  45

GAC TCT CCC GGC CAG CGT GAG CGC GCC GGC TAC GAG GAC AAG TAC GCC       192
Asp Ser Pro Gly Gln Arg Glu Arg Ala Gly Tyr Glu Asp Lys Tyr Ala
    50                  55                  60

CAG TAC GAC CAG ATC ATG TGG AAG TCG GGC GAG TTC GGC CTG ACG ACC       240
Gln Tyr Asp Gln Ile Met Trp Lys Ser Gly Glu Phe Gly Leu Thr Thr
65                  70                  75                  80
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CCG ACC AAC GGC CAA AAG TGG CTG TTT ACT GAG CCC GAG CGT GGC GTG          288
Pro Thr Asn Gly Gln Lys Trp Leu Phe Thr Glu Pro Glu Arg Gly Val
                85                  90                  95

TTC AAC TTC ACC GAG GGT GAC ATC GTG ACG AAC CTG GCC CGG AAG CAC          336
Phe Asn Phe Thr Glu Gly Asp Ile Val Thr Asn Leu Ala Arg Lys His
            100                 105                 110

GGT TTC ATG CAG CGG TGC CAC GCG CTC GTC TGG CAC AGC CAG CTC GCC          384
Gly Phe Met Gln Arg Cys His Ala Leu Val Trp His Ser Gln Leu Ala
        115                 120                 125

CCT TGG GTC GAG TCG ACC GAG TGG ACG CCC GAG GAG CTG CGC CAG GTC          432
Pro Trp Val Glu Ser Thr Glu Trp Thr Pro Glu Glu Leu Arg Gln Val
    130                 135                 140

ATT GTC AAC CAC ATC ACC CAC GTG GCC GGC TAC TAC AAG GGC AAG TGC          480
Ile Val Asn His Ile Thr His Val Ala Gly Tyr Tyr Lys Gly Lys Cys
145                 150                 155                 160

TAT GCC TGG GAC GTC GTC AAC GAG GCC CTG AAC GAG GAC GGC ACC TAC          528
Tyr Ala Trp Asp Val Val Asn Glu Ala Leu Asn Glu Asp Gly Thr Tyr
            165                 170                 175

CGC GAG TCC GTC TTC TAC AAG GTG CTC GGC GAG GAC TAC ATC AAG CTG          576
Arg Glu Ser Val Phe Tyr Lys Val Leu Gly Glu Asp Tyr Ile Lys Leu
        180                 185                 190

GCC TTC GAG ACG GCC GCC AAG GTC GAC CCC CAC GCC AAG CTC TAC TAC          624
Ala Phe Glu Thr Ala Ala Lys Val Asp Pro His Ala Lys Leu Tyr Tyr
    195                 200                 205

AAC GAC TAC AAC CTC GAG TCC CCC AGC GCC AAG ACC GAG GGC GCC AAG          672
Asn Asp Tyr Asn Leu Glu Ser Pro Ser Ala Lys Thr Glu Gly Ala Lys
210                 215                 220

CGC ATC GTC AAG ATG CTC AAG GAC GCC GGC ATC CGC ATC GAC GGC GTC          720
Arg Ile Val Lys Met Leu Lys Asp Ala Gly Ile Arg Ile Asp Gly Val
225                 230                 235                 240

GGC CTG CAG GCC CAC CTC GTC GCC GAG AGC CAC CCG ACC CTC GAC GAG          768
Gly Leu Gln Ala His Leu Val Ala Glu Ser His Pro Thr Leu Asp Glu
                245                 250                 255

CAC ATC GAT GCC ATC AAG GGC TTC ACC GAG CTC GGC GTC GAG GTC GCC          816
His Ile Asp Ala Ile Lys Gly Phe Thr Glu Leu Gly Val Glu Val Ala
            260                 265                 270

CTG ACC GAG CTC GAC ATC CGC CTC TCC ATC CCG GCC AAC GCC ACC AAC          864
Leu Thr Glu Leu Asp Ile Arg Leu Ser Ile Pro Ala Asn Ala Thr Asn
        275                 280                 285

CTC GCC CAG CAG AGG GAG GCG TAC AAG AAC GTC GTC GGC GCT TGC GTC          912
Leu Ala Gln Gln Arg Glu Ala Tyr Lys Asn Val Val Gly Ala Cys Val
    290                 295                 300

CAG GTT CGC GGC TGC ATT GGC GTG GAG ATC TGG GAC TTC TAT GAC CCC          960
Gln Val Arg Gly Cys Ile Gly Val Glu Ile Trp Asp Phe Tyr Asp Pro
305                 310                 315                 320

TTC AGC TGG GTC CCT GCC ACC TTC CCC GGC CAG GGC GCC CCC CTG CTC         1008
Phe Ser Trp Val Pro Ala Thr Phe Pro Gly Gln Gly Ala Pro Leu Leu
                325                 330                 335

TGG TTC GAG GAC TTT TCC AAG CAC CCC GCC TAC GAC GGC GTC GTC GAG         1056
Trp Phe Glu Asp Phe Ser Lys His Pro Ala Tyr Asp Gly Val Val Glu
            340                 345                 350
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GCC CTG ACC AAC AGG ACC ACG GGC GGG TGC AAG GGC AAG GGC AAG GGC      1104
Ala Leu Thr Asn Arg Thr Thr Gly Gly Cys Lys Gly Lys Gly Lys Gly
        355                 360                 365

AAG GGC AAG GTT TGG AAG GCC TAA                                     1128
Lys Gly Lys Val Trp Lys Ala
    370                 375
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 375 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met His Leu Ser Ser Ser Leu Leu Leu Ala Ala Leu Pro Leu Gly
 1               5                  10                  15

Ile Ala Gly Lys Gly Lys Gly His Gly His Gly Pro His Thr Gly Leu
            20                  25                  30

His Thr Leu Ala Lys Gln Ala Gly Leu Lys Tyr Phe Gly Ser Ala Thr
        35                  40                  45

Asp Ser Pro Gly Gln Arg Glu Arg Ala Gly Tyr Glu Asp Lys Tyr Ala
    50                  55                  60

Gln Tyr Asp Gln Ile Met Trp Lys Ser Gly Glu Phe Gly Leu Thr Thr
65                  70                  75                  80

Pro Thr Asn Gly Gln Lys Trp Leu Phe Thr Glu Pro Glu Arg Gly Val
                85                  90                  95

Phe Asn Phe Thr Glu Gly Asp Ile Val Thr Asn Leu Ala Arg Lys His
            100                 105                 110

Gly Phe Met Gln Arg Cys His Ala Leu Val Trp His Ser Gln Leu Ala
        115                 120                 125

Pro Trp Val Glu Ser Thr Glu Trp Thr Pro Glu Glu Leu Arg Gln Val
    130                 135                 140

Ile Val Asn His Ile Thr His Val Ala Gly Tyr Tyr Lys Gly Lys Cys
145                 150                 155                 160

Tyr Ala Trp Asp Val Val Asn Glu Ala Leu Asn Glu Asp Gly Thr Tyr
                165                 170                 175

Arg Glu Ser Val Phe Tyr Lys Val Leu Gly Glu Asp Tyr Ile Lys Leu
            180                 185                 190

Ala Phe Glu Thr Ala Ala Lys Val Asp Pro His Ala Lys Leu Tyr Tyr
        195                 200                 205

Asn Asp Tyr Asn Leu Glu Ser Pro Ser Ala Lys Thr Glu Gly Ala Lys
    210                 215                 220

Arg Ile Val Lys Met Leu Lys Asp Ala Gly Ile Arg Ile Asp Gly Val
225                 230                 235                 240

Gly Leu Gln Ala His Leu Val Ala Glu Ser His Pro Thr Leu Asp Glu
                245                 250                 255

His Ile Asp Ala Ile Lys Gly Phe Thr Glu Leu Gly Val Glu Val Ala
            260                 265                 270

Leu Thr Glu Leu Asp Ile Arg Leu Ser Ile Pro Ala Asn Ala Thr Asn
        275                 280                 285
```

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Leu Ala Gln Gln Arg Glu Ala Tyr Lys Asn Val Val Gly Ala Cys Val
    290                 295                 300

Gln Val Arg Gly Cys Ile Gly Val Glu Ile Trp Asp Phe Tyr Asp Pro
305                 310                 315                 320

Phe Ser Trp Val Pro Ala Thr Phe Pro Gly Gln Gly Ala Pro Leu Leu
                325                 330                 335

Trp Phe Glu Asp Phe Ser Lys His Pro Ala Tyr Asp Gly Val Val Glu
            340                 345                 350

Ala Leu Thr Asn Arg Thr Thr Gly Gly Cys Lys Gly Lys Gly Lys Gly
        355                 360                 365

Lys Gly Lys Val Trp Lys Ala
    370                 375
```

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 16, line 3-5.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany

Date of deposit	Accession Number
11 May 1995	DSM 9978

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

During the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. e.g. Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71) in those designated states providing for such "expert solution".

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

CLAIMS

1. A cloned DNA sequence encoding an enzyme exhibiting xylanase activity, which DNA sequence comprises

(a) the xylanase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9978;

(b) the DNA sequence shown in positions 1-1128 in SEQ ID NO 1 or more preferably 79-1128 or its complementary strand;

(c) an analogue of the DNA sequence defined in (a) or (b) which is at least 70% homologous with said DNA sequence;

(d) a DNA sequence which hybridizes with the DNA sequence shown in positions 1-1128 in SEQ ID NO 1 at low stringency;

(e) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with the sequences of (b) or (d), but which codes for a polypeptide having the same amino acid sequence as the polypeptide encoded by any of these DNA sequences; or

(f) a DNA sequence which is a allelic form or fragment of the DNA sequences specified in (a), (b), (c), (d), or (e).

2. The cloned DNA sequence according to claim 1, in which the DNA sequence encoding an enzyme exhibiting xylanase activity is obtainable from a microorganism, preferably a filamentous fungus, a yeast, or a bacteria.

3. The cloned DNA sequence according to claim 2, in which the DNA sequence is isolated from or produced on the basis of a DNA library of the strain *of Myceliophthora*, in particular a strain of *M. thermophila*, especially *M. thermophila*, CBS 117.65.

4. The cloned DNA sequence according to claim 2, in which the DNA sequence is obtainable from a strain of *Aspergillus*, *Trichoderma*, *Fusarium*, *Humicola*, *Neocallimastix*, *Piromyces*, *Penicillium*, *Aureobasidium*, *Thermoascus*, *Paecilomyces*, *Talaromyces*, *Magnaporthe*, *Schizophyllum*, *Filibasidium*, or a *Cryptococcus*.

5. The cloned DNA sequence according to claim 1, in which the DNA sequence is isolated from *Saccharomyces cerevisiae* DSM No. 9978.

6. A recombinant expression vector comprising a cloned DNA sequence according to any of claims 1-5.

7. A host cell comprising a cloned DNA sequence according to any of claims 1-5 or a recombinant expression vector according to claim 6.

8. The host cell according to claim 7, which is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell.

9. The host cell according to claim 8, which is a strain of *Fusarium* or *Aspergillus* or *Trichoderma*, in particular a strain of *Fusarium graminearum*, *Fusarium cerealis*, *Aspergillus niger*, *Aspergillus Oryzae*, *Trichoderma harzianum* or *Trichoderma reesei*.

10. The host cell according to claim 8, which is a strain of *Saccharomyces*, in particular a strain of *Saccharomyces cerevisiae*.

11. A method of producing an enzyme exhibiting xylanase activity, the method comprising culturing a host cell according to any of claims 7-10 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

12. An isolated enzyme exhibiting xylanase activity, characterized in (i) being free from homologous impurities and (ii) said enzyme is produced by the method according to claim 10.

13. An isolated enzyme exhibiting xylanase activity selected from the group consisting of:
(a) a polypeptide encoded by the xylanase enzyme encoding part

of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9978;

(b) a polypeptide having an amino acid sequence as shown in positions 27-375 of SEQ ID NO 2;

(c) an analogue of the polypeptide defined in (a) or (b) which is at least 70 % homologous with said polypeptide; and

an allelic form or fragment of (a), (b) or (c).

14. The enzyme according to claim 12 or 13 which has

i) a pH optimum in the range of 4-6, measured at 30°C;

ii) a molecular mass of 43 ± 10 kDa, as determined by SDS-PAGE;

iii) a temperature optimum in the range between 65°C to 75°C, measured at pH 5; and/or

iv) a specific activity in the range between 425-575 µmol/min/mg enzyme, measured with birch xylan as substrate at 30°C.

15. A composition comprising the enzyme according to any of claims 12-14.

16. An enzyme composition which is enriched in an enzyme exhibiting xylanase activity according to any of claims 12-14.

17. A composition according to claim 16, which additionally comprises a pectin lyase, pectate lyase, glucanase, xylosidase, arabinosidase, xylan acetyl esterase, or pectin methylesterase.

18. Use of an enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 in the production of dough or baked products.

19. Use of a enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 in the preparation of feed or food.

20. Use of an enzyme according to any of claims 12-14 or an

enzyme composition according any of claims 15-17 in the preparation of pulp or paper.

21. Use of an enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 for the separation of cereal components, in particular wheat components.

22. Use of en enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 for reducing the viscosity of a plant cell wall derived material.

23. Use of an enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 in the production of beer or modification of by-products from a brewing process.

24. Use of an enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 in the production of wine or juice.

25. An isolated substantially pure biological culture of the deposited strain *Saccharomyces cerevisiae* DSM No. 9978.

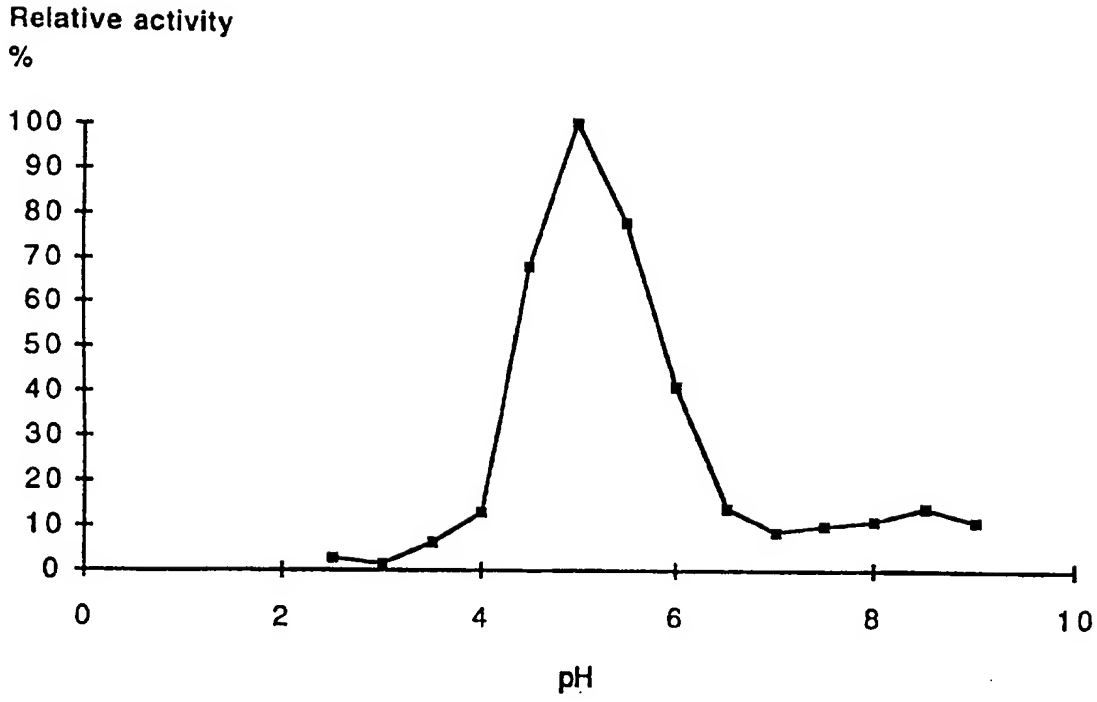

Fig. 1

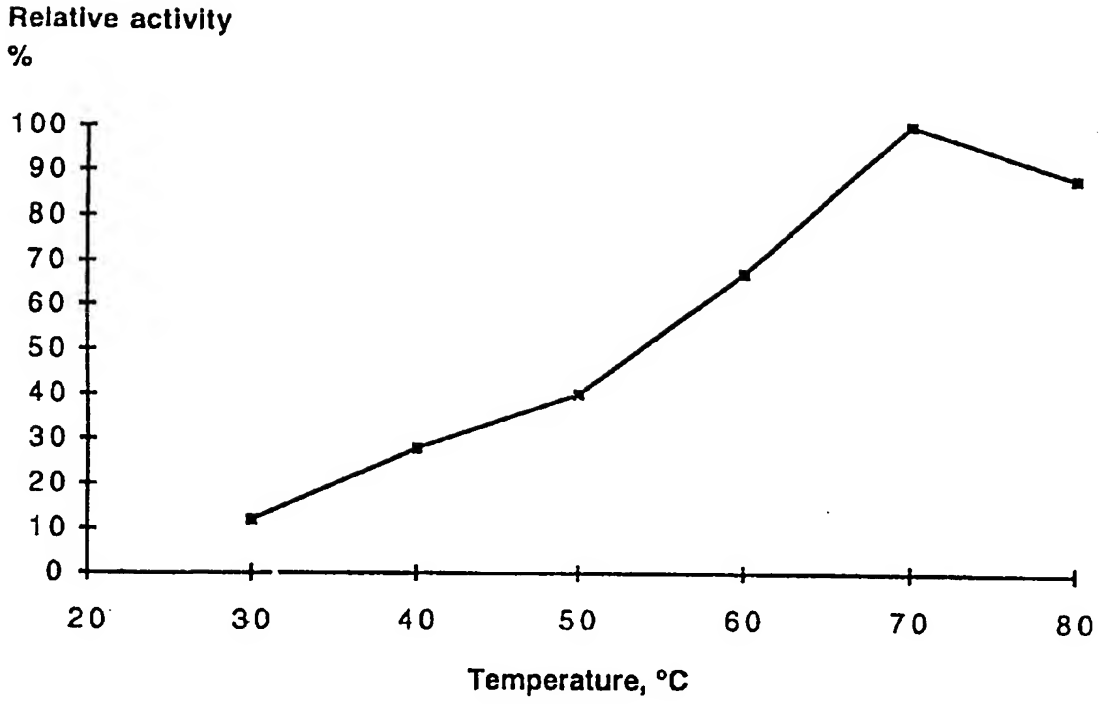
Relative activity
%
100
90
80
70
60
50
40
30
20
10
0
20
30
40
50
60
70
80
Temperature, °C

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00032

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/24 // C12S 3/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, EMBL/GENBANK/DDBJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Service, file 5, Biosis, Dialog accession no. 440901, Biosis accession no. 85041422, Dubey A.K. et al: "Xylanolytic Activity of Thermophilic Sporotrichum-SP and Yceliophthora-Thermophilum", Proc Indian Acad SCI 97 (3). 1987. 247-256 --	1-25
X	Canadian journal of microbiology, Volume 35, No 9, 1989, Ramesh K. Ganju et al, "Purification and characterization of two xylanases from Chaetomi um thermophile var. coprophile", page 836, abstract, See abstract --	1-25
X	EP 0511933 A2 (NOVO NORDISK A/S), 4 November 1992 (04.11.92), See fig 8 and claim 4 --	1-25

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 April 1997	08.05.1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00032

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chemical Abstracts, Volume 106, No 15, 13 April 1987 (13.04.87), (Columbus, Ohio, USA), Yu, Ernest K.C. et al, "Production of thermostable xylanase by a thermophilic fungus", page 500, THE ABSTRACT No 118098y, Enzyme Microb. Technol. 1987, 9 (1), 16-24 --	1-25
A	Enzyme Microb. Technol., Volume 16, April 1994, Mustafa Alam et al, "Production and characterization of thermostable xylanases by Thermomyces lanuginosus and Thermoascus aurantiacus grown on lignocelluloses", page 298, see figure 2 and figure 3 --	1-25
A	WO 9311296 A1 (VALTION TEKNILLINEN TUTKIMUSKESKUS), 10 June 1993 (10.06.93), page 9 --	1-25
A	Chemical Abstracts, Volume 116, No 19, 11 May 1992 (11.05.92), (Columbus, Ohio, USA), Oberson, Jacques et al, "Comparative investigation of cellulose-degrading enzyme systems produced by different strains of Myceliophthora thermophila", THE ABSTRACT No 190738f, Enzyme Microb. Technol. 1992, 14 (4), 303-312 --	1-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/04/97

International application No.
PCT/DK 97/00032

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0511933 A2	04/11/92	CA 2108567 A	19/10/92
		EP 0580710 A	02/02/94
		JP 6506593 T	28/07/94
		NZ 242410 A	28/03/95
		WO 9218612 A	29/10/92
WO 9311296 A1	10/06/93	AU 3088292 A	28/06/93
		BR 9206860 A	28/11/95
		CA 2125166 A	10/06/93
		EP 0615564 A	21/09/94
		FI 89613 B	15/07/93
		JP 7501587 T	16/02/95
		NO 942071 A	26/07/94